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Larkspur poisoning: toxicology and alkaloid structure–activity relationships☆

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Abstract

Systematic approaches to taxonomic classifications of the tall larkspur spp. have been developed using traditional chemical methods to profile alkaloids, comparison of relative toxicity of individual alkaloids, plant morphology/taxonomy and molecular genetics. Using these methods (papers published in this series) toxicology of three distinct species of tall larkspurs including *Delphinium glaucum*, *Delphinium barbeyi* and *Delphinium occidentale* is described. Tall larkspurs (*Delphinium* spp.) continue to be the most serious cause of cattle losses on mountain rangelands in the western US. Over 40 norditerpenoid alkaloids have been reported in species of larkspurs and toxicology data on 25 of these have been reported by the authors. These alkaloids can be classified into three general types based on their structural characteristics and toxicity: the *N*-(methylsuccinyl) anthranoyllycoctonine (MSAL)-type, having high toxicity; the lycoctonine-type, with moderate toxicity; and the 7,8-methylenedioxylycoctonine (MDL)-type, of low toxicity. The structural importance of the methylsuccinimido anthranilic acid ester group at the C18 position is evident in the high toxicity of MSAL alkaloids, particularly methyllycaconitine (MLA), Nudicauline (NUD) and 14-deacetylnudicauline (14-DAN). Other structural aspects of these alkaloids such as the C14 functionality are also important, as demonstrated by the reduced toxicity of barbinine. MLA is the alkaloid of most importance in toxicity of larkspurs on mountain rangelands because of its prevalence in most larkspurs and high toxicity. While NUD and 14-DAN also possess high toxicity, they are relatively minor components in few larkspur species (generally the plains and low larkspurs), but when present at concentrations approaching 1 mg/g dry weight they contribute significantly to overall

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toxicity. Deltaline (DLT) is often found in high concentrations in many larkspurs but because of low toxicity, its contribution to larkspur poisoning in the field is relatively minor and it will probably not cause toxicosis in the absence of the MSAL-type alkaloids. Published by Elsevier Science Ltd.

Keywords: Larkspurs; Toxicology; Norditerpenoid alkaloids; Structure–activity relationship; Deltaline; Methyllycaconitine; Nudicauline

1. Introduction

Larkspur (*Delphinium* spp.) poisoning has been a major cause of cattle losses on western rangelands for many years (Aldous, 1917; Cronin and Nielsen, 1979) and continues to be the most serious poisonous plant problem on western US high mountain rangelands (Ralphs et al. 1988, 1997). Some of the first range improvements on Forest Service allotments were 'poison fences' to keep cattle away from larkspur. Even today, the presence of tall larkspur dictates when and how some of these ranges are utilized.

Toxicology research on this problem at the Poisonous Plant Research Laboratory includes: identification and isolation of toxic alkaloids, screening for toxicity of individual alkaloids; describing alkaloid structure–activity relationships; and, elucidation of mechanism of action.

The larkspur species are generally divided into three categories: (1) tall larkspurs (*Delphinium barbeyi, Delphinium occidentale, Delphinium glaucescens, Delphinium brownii,* and *Delphinium glaucum*), 1–2 m in height, generally growing in moist high mountain habitats above 2400 m; (2) intermediate larkspurs (*Delphinium geyeri*; plains larkspur), 0.6–1 m tall and present on the short grass prairies of Wyoming, Colorado and Nebraska; and (3) low larkspurs (*Delphinium andersonii* and *Delphinium nuttallianum)* which generally grow on the desert/semidesert, foothill or low mountain ranges and are less than 0.6 m tall (Nielsen and Ralphs, 1987; Majak et al., 2000).

There is disagreement among scientists in the formal taxonomic classification of the tall larkspurs. A clear and logical description of the tall larkspurs requires morphological taxonomy (Welsh and Ralphs, 2002), genetic characterization (Li et al., 2002), chemical profiling (Gardner et al., 2002) and toxicology support. A clear definition of larkspur spp. based on these disciplines will assist land managers and livestock producers to reduce risk of poisoning to livestock.

The commonality among all the wild larkspur species is the presence of norditerpenoid alkaloids which are responsible for poisoning livestock, particularly cattle. These alkaloid toxins are of three general classes based on chemical structural features: (1) the *N*-(methylsuccinimido)-anthranoyllycoctonine (MSAL)-type; (2) the lycoctonine-type; and (3) the 7,8-methylenedioxylycoctonine (MDL)-type. These distinct structural features of the alkaloids have been correlated to their toxic activity (Manners et al., 1993; Panter et al., 1994). The MSAL-type alkaloids and in particular methyllycaconitine (MLA), nudicauline (NUD) and 14-deacetylnudicauline (14-

DAN) are the most toxic alkaloids and are responsible for the majority of toxicity of larkspurs. Deltaline (DLT), though much less toxic, is prevalent in most larkspur populations and if present in high enough concentrations it will exacerbate the toxic effects of the MSAL alkaloids. However, DLT would not be a significant threat to livestock unless toxic levels of the MSAL alkaloids are present.

This paper reviews current research on larkspur alkaloid toxicity and focuses on toxicology of individual alkaloids within defined classes, structure–activity relationships contributing to their toxicity, and their mechanism of action.

2. Toxicology

Poisoning and death losses from larkspurs primarily occur in cattle, although there are anecdotal accounts of poisoning in grazing sheep (Marsh and Clawson, 1916). In experimental dosing of cattle and sheep the progression of larkspur poisoning is the same, although sheep are 5–6 times more resistant to toxicity (Olsen, 1978, Table 1). This explains why sheep have been proposed as an alternative livestock species to graze larkspur infested ranges and are considered a potential biological control option to reduce larkspur plant cover before cattle are introduced into the pastures (Ralphs and Olsen, 1992).

While all larkspurs can cause poisoning, those species in the tall larkspur category cause the most significant losses. For example, in the western US, *D. barbeyi*, *D. occidentale* and *D. glaucum* cause significant losses to the cattle industry (Pfister et al., 1999) and in western Canada, *D. brownii* has a long history of cattle poisoning (Majak et al., 2000). Interestingly, *D. brownii* in Canada and Alaska is the same as *D. glaucum* in the Pacific States. Thus, the population in the Pacific States is referred to as *D. glaucum* (mountain larkspur) and in Alaska and Canada as *D. brownii* (tall delphinium; Majak et al., 2000). Low larkspurs (*D. nuttallianum* and *D. andersonii*) are also prevalent on many of the western US ranges and the southern interior of Canada but fewer losses occur. Alkaloid analysis of *D. brownii* compared to *D. nuttallianum* showed a 5–10-fold greater alkaloid content in *D. brownii*, thus

Table 1
Relative toxicity of DLT and MLA (mg/kg) in mice, rats, sheep and cows

Alkaloid	DLT	MLA
Mice	133	4.8
Rats	134	5.3
Sheep	50	10
Cows	50	2

Values are calculated 50% lethal dose (LD_{50}) for mice and rats. In sheep and calves the values reported are estimated 50% effective dose (ED_{50}) based on three animals. The effective dose is defined as the dose where severe clinical signs were observed but death did not occur. These are intravenous dosages with purified alkaloids (Panter, unpublished data)

explaining the difference in losses from the two species in Canada (Majak et al., 2000). While species differences in alkaloid content and concentrations are readily apparent, there are also differences depending on location, year, stage of plant growth etc. (Pfister et al., 1988; Pfister et al., 1997a,b; Ralphs et al., 2000).

Historical evidence suggested that the tall larkspur species possess different toxic properties and required individualized management practices to avoid poisoning (Aldous, 1917). Toxicological comparison of three species in a rat bioassay showed that *D. barbeyi* was most toxic and that *D. glaucescens* was four times less toxic and *D. occidentale* was 10 times less toxic than *D. barbeyi* (Olsen, 1997). Recent research using modern chemical techniques and toxicological evaluation identified the total toxic (MSAL-type) alkaloids and their relative concentrations in four tall larkspur species (Manners et al., 1995, 1998; Ralphs et al., 1997). *Delphium glaucum* collected from the Sierras was highest in toxic alkaloid concentration, *D. barbeyi* and *D. glaucescens* were intermediate and *D. occidentale* was lowest (Ralphs et al., 1997; Gardner et al., 2002).

There is apparent disagreement in taxonomic classification of the tall larkspur complex with potential misunderstanding of the toxicity of local populations. Three of these larkspur species, *D. glaucum*, *D. barbeyi* and *D. occidentale*, have similar taxonomic features and are distinguished by the type and amount of pubescence and shape of the flowers (Ewan, 1945). However, Warnock (1995) reclassified *D. occidentale* as a hybrid of *D. barbeyi* and *D. glaucum* and regrouped plants that were historically classified as *D. occidentale* and *D. glaucum*. Furthermore, the *D. barbeyi* classification was restricted to a population of plants in a small region in Utah. Therefore, a series of studies was conducted to evaluate the genetic relationships between these three species and compare their chemical profiles. Therefore, random amplified polymorphic DNA (RAPD) markers (Li et al., 2002) supported by differences in chemical profiles (Gardner et al., 2002) clearly demonstrate that *D. glaucum*, *D. barbeyi* and *D. occidentale* are separate and distinct species. Using genetic markers, chemotaxonomy and total toxic alkaloid profiles, larkspur species can be identified, toxic risk evaluated and management strategies devised to reduce larkspur poisoning on high mountain ranges.

The toxic effects of larkspurs are those of neuromuscular paralysis causing muscular weakness, muscular tremors, collapse, sternal then lateral recumbency and eventually death from respiratory failure or regurgitation aspiration (Olsen, 1978). Chronologically, signs of poisoning begin with uneasiness, stiffness of gait, an unusual characteristic straddled stance with hind limbs far apart. The animal collapses rather suddenly and usually with forelimbs buckling under first. After a variable resting period the animal may be able to stand but signs of weakness usually return quickly. Signs of nausea and abdominal discomfort are evident and vomiting may occur. If vomiting occurs, death will often result from aspiration and asphyxiation. The effects of larkspur poisoning are exacerbated by exertion and therefore, recommendations to avoid stress and disturbance are warranted.

MLA isolated from *D. brownii* was injected intravenously into calves to describe clinical response (Nation et al., 1982). Severity of clinical signs was dependant upon dosage and onset of clinical signs occurred within two to three minutes; subsequent

rapid and complete recovery with clinical signs disappearing by 10 min after injection. Calves showed signs of agitation, respiratory difficulty and loss of muscular control. Similar effects were observed in mice, rats, sheep and calves when MLA or DLT was injected i.v. (Table 1; unpublished data). When dosages of MLA and DLT were given to induce collapse, but not death, heart rate and respiration rate increased within 1–2 min after injection accompanied by uneasiness, muscular tremors and weakness before collapse. In one sheep receiving a high nonlethal dose of DLT (50 mg/kg i.v.), there was muscular weakness and collapse and one episode of grand mal-like seizures accompanied by increased heart and respiration rates 10 min after injection. Recovery occurred by 30 min, however, muscular weakness was still evident (unpublished data). In a second sheep given MLA at 10 mg/kg i.v., similar but more severe clinical signs were observed. It was believed the sheep would die without intervention, therefore neostigmine and atropine were administered i.v. two times during the most severe manifestations of toxicosis. After each injection of neostigmine and atropine, heart rate and respiration rates slowed and stabilized and subsequently the animal recovered fully. Physostigmine is known to reverse toxicosis from larkspur and MLA in cattle and has been used as an emergency treatment for poisoning (Nation et al., 1982; Pfister et al., 1994).

Larkspur alkaloids are eliminated from the blood rapidly which explains the rapid recovery after i.v. injection (Fig. 1). However, when toxicosis occurs in the field it is presumed to result from continuous consumption of larkspur and thus a slower and cumulative absorption over time. Fig. 1 illustrates preliminary data showing the blood serum elimination profile of DLT and MLA in a single sheep given a single dose of 50 mg/kg DLT or 10 mg/kg MLA i.v., respectively (unpublished data). Clinical signs of poisoning get progressively worse and appear to be cumulative over time reaching maximal effects after four consecutive days of ingestion (Olsen and Sisson, 1991).

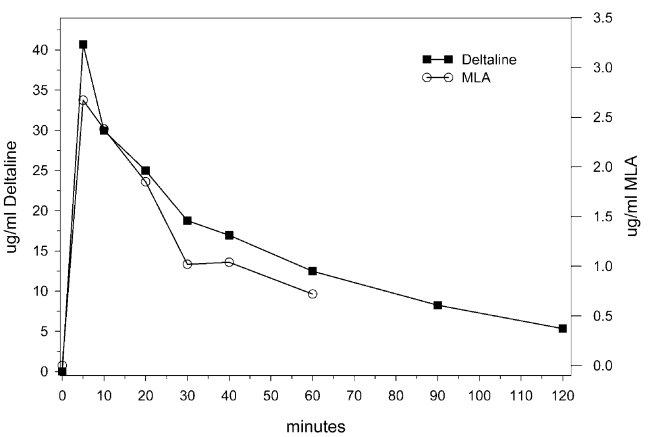


Fig. 1. Serum elimination profile from two sheep, one injected with 50 mg/kg deltaline and one injected with 10 mg/kg MLA i.v. The first phase elimination half life (t1/2) is about 20 min for both animals while the second phase t1/2 is about 70 min.

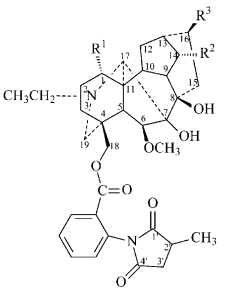
The relative risk of larkspur poisoning in the field is determined by the concentration of MSAL-type alkaloids in the plant and the propensity for cattle to eat the plant. Pfister et al. (1988) proposed a 'Toxic Window' theory based on field studies and observations. Briefly, toxicity of larkspur declines with maturity but palatability increases. Cattle generally do not graze *D. barbeyi* or *D. occidentale* in the early growth stages but consumption begins as the plants elongate flowering racemes. Consumption increases as larkspur matures into the seed pod stage. As plant growth advances to the mature pod stage the alkaloid concentration declines to a sufficiently low level that death losses become remote (Ralphs et al. 1997, 2000). Thus, for a 4–5 week period from bud elongation to mature pods, the risk of poisoning is high (Pfister et al., 1997b, 2002).

Modification of acetylcholine receptor (AchR) expression after subcutaneous nicotine injections did not increase resistance to MLA toxicity (Stegelmeier et al., 1998). Nicotine increased AchR-mRNA expression 4–5 times, but this did not have a significant impact in changing susceptibility to MLA. While low level feeding of larkspur diets containing 1.1 or 4.4% larkspur was believed to impart tolerance to poisoning, it actually appeared to exacerbate the toxicity of MLA.

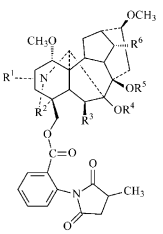
Use of cholinesterase inhibitors showed some benefit in reducing toxicity and preventing death from larkspur poisoning. In rat studies, administration of the cholinesterase inhibitors/antagonists, fenthion, famphur, physostigmine, neostigmine and neostigmine/glycopyrrolate showed beneficial effects in treatment of acute MLA toxicity (Stegelmeier et al., 1998). Intravenous injections of physostigmine to intoxicated steers in experimental settings prevented death, however, repeated injections were required (Pfister et al., 1994). Administering physostigmine or neostigmine in the field to a lethally poisoned cow is difficult and impractical because one would need to treat the animal soon after showing recumbency to prevent death. Also, as GI absorption of alkaloids from oral ingestion of larkspur continues, one may need to repeat treatment with physostigmine over the intoxication period to prevent death. In the field, recumbent animals often recover spontaneously without treatment but survival frequently depends on dose and posture of the recumbent animal to allow eructation. Further research is needed to demonstrate a practical and effective field treatment useful to ranchers.

Attempts at experimental modification of larkspur toxicity using mouse and rat bioassays have been variable (Stegelmeier et al., 1998). Larkspur alkaloids are apparently not metabolized by the P450 hepatic enzymes since phenobarbital (inducer) or SKF 525-A (inhibitor) did not alter toxicity. However, animals that were clinically depressed by phenobarbital seemed most resistant. Subsequently, rats were given Valium treatments and they showed a trend towards increased tolerance for MLA toxicity (Stegelmeier et al., 1998). This research further supports the idea that poisoned animals should not be stressed.

Over 40 norditerpenoid alkaloids have been reported in species of larkspurs and toxicity data in a mammalian system for 25 of these have been reported by the authors (Figs. 2–4; Manners et al., 1993, 1995, 1998; Gardner et al., 2000). Based on alkaloid analysis and toxicity testing, four alkaloids are probably responsible for the majority of tall larkspur toxicity namely, MLA, NUD and 14-DAN, of the



Alkaloid	Functionality			Relative Toxicity	LD_{50} in Mouse Bioassay
	R^1	R^2	R^3		
Methyllycoconitine (MLA)	OCH_3	OCH_3	OCH_3	1	4.8
Nudicauline	OCH_3	OAc	OCH_3	0.6	2.7
14-Acetylbearline	OCH_3	OAc	OAc	0.7	3.3
14-Deacetylnudicauline	OCH_3	OH	OCH_3	0.8	4.0
Bearline	OCH_3	OH	OAc	1.2	5.7
Geyerline	OCH_3	OCH_3	OAc	1.3	6.2
Grandiflorine	OH	OCH_3	OCH_3	11.7	8.5
Barbinine	OCH_3	=O	OCH_3	11.9	57
Grandiflorine acetate*	OAc	OCH_3	OCH_3	20.8	100

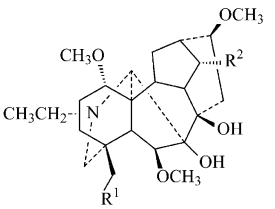


Alkaloid	Functionality						Relative Toxicity	LD_{50} in Mouse Bioassay
	R^1	R^2	R^3	R^4	R^5	R^6		
Elatine	CH_3CH_2	H_2	OCH_3	CH_2	CH_2	OCH_3	1.9	9.2
N-Desethyl MLA	H	H_2	OCH_3	H	H	OCH_3	20.8	100
Anhweidelphinine		H	OCH_3	H	H	OCH_3	36.9	> 177
Zaliline	H	=O	OCH_3	H	H	OCH_3	50	240

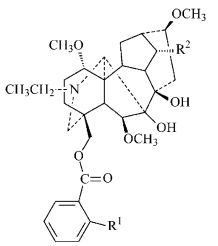
Fig. 2. MSAL [*N*-(methylsuccinyl) anthranoyllycoctonine]-type norditerpenoid alkaloids. Chemical functionalities with relative toxicity compared to MLA and mouse bioassay LD50s are tabulated. Note: Relative toxicity of MSAL alkaloids is compared to MLA which is assigned the number 1. For example, nudicauline is 0.6 times more toxic than MLA while zaliline is 50 times less toxic.

MSAL-type, and DLT of the MDL-type. Although DLT belongs to the MDL-type alkaloids of low toxicity, the high concentration (10–30 mg/g) found in many populations of larkspur plants makes it a significant threat contributing to overall toxicity and losses. However, the contribution of DLT to larkspur poisoning is relatively minor and toxicosis will not occur in the absence of significant levels (>3 mg/g dry weight) of MSAL-type alkaloids.

Variations in structural features of each norditerpenoid alkaloid can exacerbate or reduce toxicity. The toxicological impact of these structural aspects will be discussed later in this paper.



Alkaloid	Functionality		Relative Toxicity	LD_{50} in Mouse Bioassay
	R^1	R^2		
Anthranoyllycoctonine		OCH_3	4.4	21
14-Dehydrobrowniine*	OCH_3	=O	52.9	> 254
Lycoctonine	OH	OCH_3	93.5	449
Browniine (76%)*	OCH_3	OH	150	> 720

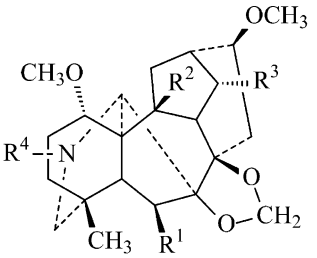


Alkaloid	Functionality		Relative Toxicity	LD_{50} in Mouse Bioassay
	R^1	R^2		
Delavaine A + B	$NHCOCH(CH_3)CH_2COOCH_3$ (A) $NHCOCH_2CH(CH_3)COOCH_3$ (B)	OCH_3	0.7	3.3
Anthranoyllycoctonine	NH_2	OCH_3	4.4	21
Andersonine*	$NHCOCH(CH_3)CH_2COOCH_3$ or $NHCOCH_2CH(CH_3)COOCH_3$	OAc	5.2	25

Fig. 3. Lycoctonine-type norditerpenoid alkaloids. As in Fig. 2, relative toxicity is compared to MLA.

3. Mechanism of action

The early clinical signs of larkspur poisoning in cattle described in the 1950s suggested the effects resulted from neuromuscular blockade (Dozortseva-Kubanova, 1959; Aiyar et al., 1979). It is now known that the MSAL-type alkaloids are potent neuromuscular poisons in mammals, acting at the post-synaptic neuromuscular junction (Benn and Jacyno, 1983). In mice and rats, high doses of MLA also elicit central nervous system (CNS) effects (Dozortseva-Kubanova, 1959; Stegelmeier et al.,



Alkaloids	Functionality				Relative Toxicity	LD_{50} in Mouse Bioassay
	R^1	R^2	R^3	R^4		
Deltaline	OAc	OH	OCH_3	CH_2CH_2	27.7	133
Deltamine*	OH	OH	OCH_3	CH_3CH_2	31.3	150
N-Desethyldeltaline	OAc	OH	OCH_3	H	43.8	210
Dictyocarpine	OAc	OH	OH	CH_3CH_2	59.0	283
14-OAc Dictyocarpine*	OAc	OH	OAc	CH_3CH_2	159.4	> 765

Fig. 4. MDL (7,8-methylenedioxylycoctonine)-type norditerpenoid alkaloids. Again, as in Fig. 2, relative toxicity is compared to MLA.

1998). The neurotoxic clinical signs of MLA and other norditerpenoid alkaloids were described as classical curare-like (Benn and Jacyno, 1983). These alkaloids compete as post-synaptic inhibitors of the neurotransmitter acetylcholine specifically acting at the α1 nicotinic sites (Dobelis et al., 1999). MLA is a potent competitor of α-bungarotoxin (α-BGT) binding tightly to the nicotinic acetylcholine receptors (nAChR) at the nanomolar range in insects (Jennings et al., 1986). Recent comparisons of binding affinities of MSAL and MDL alkaloids using lizard muscle nAChR demonstrated distinct differences in binding affinities (Dobelis et al., 1999). Differences in binding affinity and toxicity among MSAL norditerpenoid alkaloids and between MSAL and MDL alkaloids are apparently related to structural characteristics of the alkaloids i.e. NUD>14-DAN>MLA>>barbinine>>>>DLT (Figs. 2–4; Table 2).

A competitive binding assay using I^{125}-labeled α-bungarotoxin was used to determine the binding of the larkspur alkaloids to rat and cow brain and skeletal muscle AchR (Rapier et al., 1985; Macallan et al., 1988). Comparing these binding affinities with those reported in the literature shows a strong likelihood that variations in species susceptibility to larkspur poisoning are related to binding affinity of the alkaloids to the receptor site (Ward et al., 1990; Stegelmeier et al., 1998). This binding affinity may explain the variation in susceptibility and manifestation of clinical signs of poisoning by MLA between species i.e. cattle>mice = rats>sheep (Table 1; unpublished data). There are also apparent species differences in the clinical signs of poisoning. Thus, cattle show severe skeletal muscle weakness as opposed to the

Table 2
IC_{50}[a] values of CMAP in lizard muscle and concentration of alkaloids to inhibit MEPP compared to LD_{50} values in a mouse bioassay for four MSAL and one MDL (DLT) alkaloid

	IC_{50}[b]		LD_{50} in mice (mg/kg)
	CMAP	MEPP (μm)	
MLA	1.48	0.10	4.8[a]
Nudicauline	0.32	–	2.7
14-DAN	0.62	0.05	4.0
Barbinine	13.2	0.50	57
Deltaline	156	20	159.4

Table adapted from Manners et al., 1998; Dobelis et al., 1999 and Panter unpublished data

[a] Data previously published by the authors indicated that the LD_{50} of MLA in a mouse bioassay was 7.5 mg/kg (Manners et al., 1998), however, more recent unpublished data using ultra pure material determined the LD_{50} to be 4.8 mg/kg.

[b] IC_{50} is the alkaloid concentration inducing 50% inhibition.

additional convulsion-like signs in the mouse and rat. These differences are reflected in the CNS-AchR binding affinities, since rats had nearly 100× higher binding affinity to MLA than cattle (Stegelmeier et al., 1998). The toxic MSAL alkaloids such as NUD, 14-DAN and MLA had higher binding affinites than the less toxic MSAL alkaloid barbinine and the MDL alkaloid, DLT (Dobelis et al., 1999; Table 2).

The pharmacology of MLA as the hydroiodide salt (mellictine) was published by Dozortseva-Kubanova (1959) and reviewed by Benn and Jacyno (1983). MLA has a marked effect on skeletal muscle vs little or no effect on cardiac muscle. Dosing mice with 2 mg/kg i.v. mellictine produced muscular relaxation, respiratory depression and clonic/tonic convulsions with return to normal after 15–20 min. Larger doses of 3.0–3.5 mg/kg caused complete motor paralysis, respiratory arrest and death. Similar effects were produced when MLA was given orally or rectally (Dozortseva-Kubanova, 1959). No cardiac abnormalities were observed. In experiments on anesthetized cats, 1 mg/kg i.v. induced a brief fall in blood pressure and higher doses caused hypotension but no significant changes in cardiac rhythm or amplitude of the heart signal (Dozortseva-Kubanova, 1959). Subsequently, perfusion of a media solution containing concentrations of 1×10^{-6}–2.5×10^{-6} M mellictine into blood vessels of the isolated rabbit ear, kidney or heart produced no vascular changes. Mellictine also had no effect on smooth muscle of the isolated rabbit intestine, guinea pig uterus or guinea pig ileum at concentrations up to 5×10^{-6} M, suggesting limited or no muscarinic receptor activity.

As previously discussed, MLA is active at the CNS level, especially in rodents where seizure-like effects occur after large i.v. injections. However, those seizures are not common in cattle or sheep suggesting physiological differences in the blood brain barrier and or binding affinity to receptor sites, in the CNS between livestock and rodents. One of the documented differences is binding affinity of MLA to CNS-AchR. Binding affinity in the brain is 100× greater in rats compared to cows

(Stegelmeier et al., 1998). Experiments using fetal rat hippocampal tissue in culture demonstrated that MLA antagonizes acetylcholine in a dose-dependant but reversible manner (Alkondon et al., 1992). MLA was also shown to compete with α-bungarotoxin binding to the rat hippocampal membrane nAchR with a higher binding affinity for brain sites in the rat than muscle sites (Ward et al., 1990). The MSAL-type alkaloids: MLA, 14-DAN, elatine, glaudelsine and elanine were the most potent binding inhibitors in the rat neuronal tissues with MLA>elatine>14-DAN>elanine>glaudelsine, while three MDL alkaloids and nine other larkspur alkaloids showed much lower binding affinity (Kukel and Jennings, 1994).

Ganglionic blocking properties of MLA were shown using the nictitating membrane preparation of the cat with a threshold dosage at 1–2 mg/kg i.v. and complete inhibition at 4 mg/kg. This same dosage also blocked cardiac muscle response to vagus nerve electrical stimulation (Dozortseva-Kubanova, 1959). The hypotensive effects at high doses were also attributed to the ganglion blocking effect of MLA.

The most pronounced effects of MLA are on skeletal muscle, especially in cattle. Injections of 13–14 mg/kg of mellictine into the abdominal lymph sac of the frog completely blocked contractile response of the gastrocnemius muscle when electrical stimulation was applied to the sciatic nerve (Dozortseva-Kubanova, 1959). The same effects were reported in vitro when isolated frog muscle preparations were bathed in 2.5×10^{-5} M concentrations of mellictine. This same concentration blocked the response of the isolated frog rectus abdominis muscle to acetylcholine. Using frog Sartorius muscle preparations, Jacyno (1981) and Benn and Jacyno (1983) demonstrated 80–90% inhibition of post-synaptic recorded action potentials upon sciatic nerve stimulation after muscle tissue was bathed for 2 min in 10^{-8} M MLA. Direct electrical stimulation to the muscle elicited normal contractions. Solutions containing 10^{-7} M MLA caused complete inhibition which was partially reversed when physostigmine was introduced at 10^{-5} M. Inhibition in the amplitude of miniature endplate potential (MEPP) of the frog extensor digitus longus IV muscle occurred at MLA concentrations of 10^{-7} M and complete inhibition at 4×10^{-6} M (Benn and Jacyno, 1983). Similar results were observed using a rat phrenic nerve-diaphragm preparation with 50% inhibition at 2×10^{-5} M and complete inhibition at 3×10^{-5} M. The MLA-inhibited diaphragm muscle responded normally when stimulated directly and the inhibition was partially reversed with physostigmine (Aiyar et al., 1979).

In anesthetized cats doses of 2 mg/kg i.v. of MLA elicited threshold inhibition of the sciatic nerve-gastrocnemius muscle preparation. At 3 mg/kg a sharp decreased response to electrical stimulation in muscle contractions occurred gradually returning to normal in 20–25 min but 3.5 mg/kg i.v. completely blocked muscle response resulting in death. This neuromuscular block was reversed by i.v. administration of 0.15 mg/kg neostigmine.

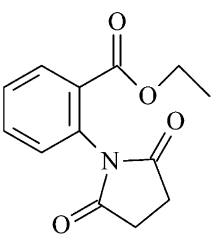
These experiments demonstrate that the norditerpenoid alkaloids from larkspurs are neuromuscular toxins, inhibiting normal signal transmission by acetylcholine across the neuromuscular junction. Intensity of this inhibition is associated with specific structural characteristics of the alkaloids.

4. Structure–activity relationships

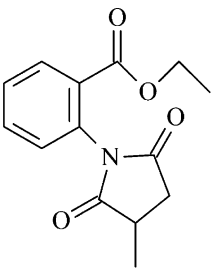
While the mechanism of action of the norditerpenoid alkaloids involves blocking of neuromuscular transmission at the α1 nicotinic acetylcholine receptors, relative toxicity of individual alkaloids is observed to change with variations in the structural character of the alkaloids (Manners et al., 1995; Dobelis et al., 1999). In comparison with the lycoctonine and MDL-type alkaloids, the high toxicity of the three MSAL-type alkaloids (NUD, 14-DAN, MLA) can be associated with the presence of a methylsuccinylanthranoyl ester at C18. It is interesting to note that removal of this ester functionality from MLA thereby forming lycoctonine, destroys neuromuscular blocking action on the frog sciatic nerve Sartorius muscle preparation (Aiyar et al., 1979). Lycoctonine is still toxic in mammals but almost 100-fold less so than MLA. However, the C18 functionality is not the only component affecting binding affinity. Differences among the MSAL-type alkaloids in their binding affinity at the α1 receptors and thus their toxicity, are apparently also affected by the C14 functionality (Fig. 2; Manners et al., 1993, 1995, 1998; Dobelis et al., 1999). These differences are demonstrated by the relatively low toxicity of barbinine compared to MLA whereby a carbonyl function replaces a methoxy group at C14. Comparison of toxicity in a mouse bioassay for MLA, NUD, 14-DAN and barbinine with in vitro results of binding affinity to lizard muscle nAChR further confirms the importance of the oxidation state and functionality at C14 (Manners et al., 1995; Dobelis et al., 1999). Table 2 compares the effects of these four alkaloids and DLT on compound muscle action potential (CMAP) and MEPP using the lizard muscle preparation. Similar relationships exist between these alkaloids when toxicity was compared using a mammalian system, i.e. mouse bioassay (Manners et al., 1995).

Other sites on the norditerpenoid alkaloids are also important and various functionalities at those sites may enhance or reduce toxicity (Manners et al. 1995, 1998; Gardner et al., 2000). Toxicological data for other norditerpenoid alkaloids (Figs. 1–3) provide evidence of the importance of a tertiary alkaloid nitrogen, substitution of anthranilic acid moiety and character of substitutions at C1, to the toxicity of these compounds.

When the methylsuccinylanthranoyl ester is cleaved from MLA and each component tested separately in the mouse bioassay, the results are interesting. Lycoctonine (MLA with the anthranilic ester removed) induced similar clinical response in mice to that of MLA when given i.v., but was 93 times less toxic than MLA (LD_{50} lycoctonine=449 mg/kg vs LD_{50} MLA=4.5 mg/kg i.v.; Figs. 2 and 3). When the toxicity of the succinic anhydride (SA) or the methyl succinic anhydride (MSA) of the methylsuccinyl anthranilic ester moiety was tested in mice the clinical response was dramatically different (Fig. 5; unpublished data). No tremors were induced, neuromuscular stimulation was absent and toxicity was relatively low (LD_{50} SA=438 mg/kg; LD_{50} MSA=163 mg/kg). Therefore, the most obvious functionality whereby NUD, MLA and 14-DAN differ from the weakly active alkaloid, lycoctonine, is the complex ester grouping at C18 (Fig. 1) as discussed. Evaluation of toxicity data (Figs. 2–4) establishes two critical structural features (an *N*-ethyl bicyclo substi-



Succinic Anhydride (SA) of
Anthranilic Acid Functionality
LD_{50} = 438 mg/kg



Methyl Succinic Anhydride (MSA)
of Anthranilic Acid Functionality
LD_{50} = 165 mg/kg

Fig. 5. Anthranilic acid functionality of MLA without which the MSAL alkaloids would be much less toxic. Note the increased toxicity of the methyl derivative.

tuted tertiary alkaloid nitrogen atom and C18 anthranilic acid ester) as necessary to impart high toxicity to these larkspur alkaloids (Taylor, 1990; Manners et al., 1995).

The active core of the norditerpenoid alkaloids is the lycoctonine skeleton associated with interference of the cholinergic neurotransmission. The quaternary amine element of all the diterpene alkaloids is an important component of the neuromuscular blocking effects, as also is the case with curare, decamethonium, etc. (Taylor, 1990). As already stated, the methylsuccinylanthranoyl ester at C18 imparts potency to the alkaloids via orientation and or binding affinity to the nicotinic receptors. However, the C14 functionalities and the pattern of oxygenation and the electronic nature of the oxygen bearing functionalities appear to enhance the physiological manifestations and potency of toxicity. This concept explains the structural component of the most potent competitive antagonists of acetylcholine at the skeletal neuromuscular junction among the norditerpenoid alkaloids mainly, NUD, MLA and 14-DAN.

Jacyno (1981) compared space-filling molecular models of MLA with (+)-tubocurarine chloride and found similarity in overall size and conformation. It appeared that MLA fits the ‘curariform template’ described by Pauling and Petcher and reported by Jacyno (1981). The importance of the C18 functionality was further elaborated by measuring and comparing the effects of MLA, anthranoyllycoctonine and lycoctonine using the MEPP in an extensor digitus longus IV muscle preparation from *Rana pipens*. It was demonstrated that anthranoyllycaconitine was 0.1×, and lycoctonine about 0.02×, as potent as MLA. Therefore, Jacyno (1981) concluded that the ester function in MLA provides secondary binding sites or facilitates an essential orientation of the alkaloid to the receptor site. Therefore, the key acetylcholine receptor ‘recognition sites’ on the norditerpenoid alkaloids are apparently the nitrogen and the C18 oxygen.

New knowledge about structural features of the larkspur alkaloids has enhanced understanding about larkspur poisoning, and has helped identify those alkaloids of highest risk within larkspur species. The toxicological testing of future MSAL-related

norditerpenoids obtained from larkspurs or synthetic analogs will continue to expand our knowledge about the mode of action of these toxins.

5. Systematic and ecological approach to prevention of poisoning

For both theoretical and practical reasons, the structural features of norditerpenoid larkspur alkaloids that impart toxic potency are significant when evaluating toxicity and relative grazing risk of larkspur species or populations. The level of MLA generally accounts for the difference in toxicity between the tall larkspur spp. By measuring the concentration of the toxic alkaloids in larkspurs, and even within populations, the potential risk of toxicity can be quantified. *D. glaucum* had the highest concentration of toxic alkaloids exceeding 3 mg/g until maturity and *D. barbeyi* is intermediate where the concentration of toxic alkaloids typically declines below 3 mg/g during the pod stage. Some populations of *D. occidentale*, and its hybrids with *D. barbeyi* do not contain toxic alkaloids and the concentration of toxic alkaloids in northern populations of *D. occidentale* varied year to year.

Larkspur populations should be tested during the flowering and pod stages of growth. If the MSAL alkaloid concentration exceeds 3 mg/g during the flowering stage when cattle often initiate consumption, cattle should be removed until pods shatter later in the season. Likewise, when the concentration of the MSAL alkaloids exceeds 3 mg/g in the pods, risk will be moderate to high if cattle are eating the pods. Larkspurs containing lower concentrations of the MSAL alkaloids ($<$3 mg/g) in the leaves, flowers or pods should be safe for grazing throughout the grazing season because cattle would need to ingest very large quantities for fatalities to occur.

6. Conclusion

While much is now known about identifying high risk pastures for larkspur poisoning, the mechanism of toxic action, structure–activity relationship of toxic alkaloids, and grazing behavior surrounding larkspur ingestion, we have yet to completely eliminate death losses in cattle. Management of livestock is still the most practical method to reduce animal losses. As technology advances and enhanced understanding of animal/plant/environmental interactions becomes more fully understood, further reductions in cattle losses will be accomplished. Further use of new technologies including gene expression or qualitative loci analysis in cattle to select resistance, coupled with information about risk of poisoning, will certainly play a future role in reducing or preventing cattle losses on larkspur infested pastures. Further studies will focus on alkaloid interactions, development of immune based diagnostic techniques and the prevention of poisoning via vaccines, pharmacological products, selection of resistant animals and grazing management.

References

Aiyar, V.N., Benn, M.H., Hanna, T., Jacyno, J.M., Roth, S.H., Wilkens, J., 1979. The principal toxin of *Delphinium brownii* Rydb., and its mode of action. Experientia 35, 1367–1368.

Alkondon, M., Pereira, E.F.R., Wonnacott, S., Albuquerque, E.X., 1992. Mol. Pharmacol. 41, 802–806.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Benn, M.H., Jacyno, J.M., 1983. The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier, S.W. (Ed.), Alkaloids, Chemical and Biological Perspectives. Wiley, New York, pp. 153–211.

Cronin, E.H., Nielsen, D.B., 1979. The ecology and control of rangeland larkspurs. UT. Agr. Exp. Sta. Bull. 499, Utah State University, Logan, UT.

Dobelis, P., Madl, J.E., Pfister, J.A., Manners, G.D., Walrond, J.P., 1999. Effects of *Delphinium* alkaloids on neuromuscular transmission. J. Pharmacol. Exp. Ther. 291, 538–546.

Dozortseva-Kubanova, P.M., 1959. The Pharmacology of the alkaloid methyllycaconitine (mellictine). Farmakol. Toksikol. 22, 30–34.

Ewan, J., 1945. A synopsis of the North American species of *Delphinium*. Univ. Colo. Studies Ser. D. 2, 1–244.

Gardner, D.R., Manners, G.D., Panter, K.E., Lee, S.T., Pfister, J.A., 2000. Three new toxic norditerpenoid alkaloids from the low larkspur *Delphinium nuttallianum*. J. Nat. Prod. 63, 1127–1130.

Gardner, D.R., Turner, D., Ralphs, M.H., Welsh, S., 2002. Taxonomic implications of diterpene alkaloids in three toxic tall larkspur species (*Delphinium* spp.). Biochem. Syst. Ecol. 30, 77–90.

Jacyno, J.M., 1981. PhD thesis, University of Calgary.

Jennings, K.R., Brown, D.G., Wright, D.P. Jr., 1986. Experientia 35, 611.

Kukel, C.F., Jennings, K.R., 1994. *Delphinium* alkaloids as inhibitors of alpha-bungarotoxin binding to rat and insect neural membranes. Can. J. Physiol. Pharmacol. 72, 104–107.

Li, X., Ralphs, M.H., Gardner, D.R., Wang, R.R.C., 2002. Genetic variation within and among 22 accessions of three tall larkspur species (*Delphinium*) spp. based on RAPD markers. Biochem. Syst. Ecol. 30, 91–102.

Macallan, D.R., Lunt, G.G., Wonnacott, S., Swanson, K.L., Rapoport, H., Albuquerque, E.X., 1988. Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems. FEBS Lett. 226, 357–363.

Majak, W., Mcdiarmid, R.E., Hall, J.W., Willms, W., 2000. Alkaloid levels of a tall larkspur species in southwestern Alberta. J. Range Manage. 53, 207–210.

Manners, G.D., Panter, K.E., Ralphs, M.H., Pfister, J.A., Olsen, J.D., James, L.F., 1993. Toxicity and chemical phenology of norditerpenoid alkaloids in the tall larkspurs (*Delphinium* species). J. Agric. Food Chem. 41, 96–100.

Manners, G.D., Panter, K.E., Pelletier, S.W., 1995. Structure–activity relationships of norditerpenoid alkaloids occurring in toxic larkspur (*Delphinium*) species. J. Nat. Prod. 58, 863–869.

Manners, G.D., Panter, K.E., Pfister, J.A., Ralphs, M.H., James, L.F., 1998. The characterization and structure–activity evaluation of toxic norditerpenoid alkaloids from two *Delphinium* species. J. Nat. Prod. 61, 1086–1089.

Marsh, C.D., Clawson, A.B., 1916. Larkspur poisoning of livestock. USDA Bulletin No. 365.

Nation, P.N., Benn, M.H., Roth, S.H., Wilkens, J.L., 1982. Clinical signs and studies of the site of action of purified larkspur alkaloid, methyllycoconitine, administered parenterally to calves. Can. Vet. J. 23, 264–266.

Nielsen, D.B., Ralphs, M.H., 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, CO, pp. 119–129.

Olsen, J.D., 1997. Toxicity of extract from three larkspur species (*Delphinium barbeyi*, *D. glaucescens*, and *D. occidentale*) measured by rat bio-assay. J. Range Manage. 30, 237–238.

Olsen, J.D., 1978. Tall larkspur poisoning in cattle and sheep. Am. Vet. Med. Assoc. 173, 762–765.

Olsen, J.D., Sisson, D.V., 1991. Description of a scale for rating the clinical response of cattle poisoned by larkspur. Am. J. Vet. Res. 52, 488–493.

Panter, K.E., Manners, G.D., James, L.F., Pfister, J.A., Ralphs, M.H., Gardner, D.R., Stegelmeier, B.L.,

Olsen, J.D., 1994. Toxicity of larkspur (*Delphinium* spp.) in cattle. Proc. Western Section ASAS 45, 95–97.

Pfister, J.A., Gardner, D.R., Price, K.W., 1997a. Grazing risk on tall larkspur-infested rangelands. Rangelands 19, 12–15.

Pfister, J.A., Gardner, D.R., Panter, K.E., Manners, G.D., Ralphs, M.H., Stegelmeier, B.L., Schoch, T.K., 1999. Larkspur (*Delphinium* spp.) poisoning in livestock. J. Nat. Toxins 8, 81–94.

Pfister, J.A., Manners, G.D., Ralphs, M.H., Hong, Z.X., Lane, M.A., 1988. Effects of phenology, site, and rumen fill on tall larkspur concumption by cattle. J. Range Manage. 41, 509–514.

Pfister, J.A., Ralphs, M.H., Gardner, D.R., Stegelmeier, B.L., Manners, G.D., Panter, K.E., Lee, S.T., 2002. Management of three toxic Delphinium species based on alkaloid concentration. Biochem. Syst. Ecol. 30, 129–138.

Pfister, J.A., Panter, K.E., Manners, G.D., 1994. Effective dose in cattle of toxic alkaloids from tall larkspur (*Delphinium barbeyi*). Vet. Hum. Toxicol. 36, 511–514.

Pfister, J.A., Ralphs, M.H., Manners, G.D., Gardner, D.R., Price, K.W., James, L.F., 1997b. Early season grazing by cattle of tall larkspur (*Delphinium* spp.) infested rangeland. J. Range Manage. 50, 391–398.

Ralphs, M.H., Gardner, D.R., Pfister, J.A., 2000. A functional explanation for patterns of norditerpenoid alkaloid levels in tall larkspur (*Delphinium barbeyi*). J. Chem. Ecol. 26, 1595–1607.

Ralphs, M.H., Manners, G.D., Pfister, J.A., Gardner, D.R., James, L.F., 1997. Toxic alkaloid concentration in tall larkspur species in the western United States. J. Range Manage. 50, 497–502.

Ralphs, M.H., Olsen, J.D., 1992. Prior grazing by sheep reduces waxy larkspur consumption by cattle: an observation. J. Range Manage. 45, 136–139.

Ralphs, M.H., Olsen, J.D., Pfister, J.A., Manners, G.D., 1988. Plant–animal interactions in larkspur poisoning in cattle. J. Anim. Sci. 66, 2334–2342.

Rapier, C., Harrison, R., Lunt, G.G., Wonnacott, S., 1985. Neosurugatoxin blocks nicotinic acetylcholine receptors in the brain. Neurochem. Int. 7, 389–396.

Stegelmeier, B.L., Panter, K.E., Pfister, J.A., James, L.F., Manners, G.D., Gardner, D.R., Ralphs, M.H., Olsen, J.D., 1998. Experimental modification of larkspur (*Delphinium* spp.) toxicity. In: Garland, T., Barr, C. (Eds.), Toxic Plants and Other Natural Toxicants. CAB International, New York, pp. 205–210.

Taylor, P., 1990. Agents acting at the neuromuscular junction and autonomic ganglia. In: Gilman, A.G., Rall, T.W., Nies, A.S., Taylor, P. (Eds.), The Pharmacological Basis of Therapeutics. Pergamon Press, New York, pp. 166–186.

Ward, J.M., Cockcroft, V.B., Lunt, G.G., Smillie, F.S., Wonnacott, S., 1990. Methyllycaconatine: a selective probe for neuronal alpha-bungarotoxin binding sites. FEBS Lett. 270, 45–48.

Warnock, M.J., 1995. A taxonomic conspectus of North American *Delphinium*. Phytologia 78, 73–101.

Welsh, S.E., Ralphs, M.H., 2002. Some tall larkspurs (*Delphinium* — Ranunculacea), a taxonomic review. Biochem. Syst. Ecol. 30, 103–112.